MANGALORE UNIVERSITY

ISOLATION AND CHARACTERIZATION OF PISCEAN COLLAGEN AND ITS BIOLOGICAL APPLICATIONS

Synopsis of Ph.D. Thesis

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The thesis entitled **"ISOLATION AND CHARACTERIZATION OF PISCEAN COLLAGEN AND ITS BIOLOGICAL APPLICATIONS"** deals mainly with the isolation, characterization and applications of isolated collagen-based composites from Indian oil sardine and Indian mackerel. The work is divided into five chapters.

CHAPTER I: A BRIEF REVIEW OF LITERATURE ON ISOLATION, CHARACTERIZATION AND APPLICATIONS OF PISCEAN COLLAGEN

CHAPTER II: ISOLATION OF COLLAGEN FROM INDIAN OIL SARDINE (*Sardinella longiceps*) AND ITS BIOCHEMICAL CHARACTERIZATION

CHAPTER III: ISOLATION OF COLLAGEN FROM INDIAN MACKEREL (*Rastrelliger kanagurta*) AND ITS BIOCHEMICAL CHARACTERIZATION

CHAPTER IV: STUDIES ON WOUND CLOSURE APPLICATIONS OF ISOLATED ACID-SOLUBLE COLLAGEN/NANOMETAL OXIDE COMPOSITES

CHAPTER V: FABRICATION OF ENVIRONMENTALLY COMPATIBLE BIOPOLYMER FILMS OF PULLULAN/PISCEAN COLLAGEN/ZnO NANOCOMPOSITE AND THEIR ANTIMICROBIAL ACTIVITY

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CHAPTER I

A BRIEF REVIEW OF LITERATURE ON ISOLATION, CHARACTERIZATION AND APPLICATIONS OF PISCEAN COLLAGEN

The fish industry is a major economic source for many countries worldwide. Fish protein is a vital source of nutrients for many people, especially in developing countries. It is estimated that worldwide one billion people depend on producing, processing, and trading fish for their livelihood [1]. The fish processing industry produces more than 60 % of by-products as waste, which includes head, skin, trimmings, fins, frames, viscera, roes and only 40 % of fish products used for human consumption [2]. Large quantities of fish by-product wastes from fisheries would create serious environmental pollution, create offensive odors and leads to disposal problems in both developed and developing countries. These wastes contain a good amount of protein-rich materials that were normally processed into low market-value products, such as animal feed, fishmeal, fish oil, pet food, fish silage and fertilizer. So, substantial amount of research had been carried out to develop methods to convert these wastes into value-added products. The isolation of bioactive compounds from these wastes would solve the environmental pollution and disposal problems [3].

The proteins in fish could be divided into the following three groups:

(i) Structural proteins (actin, myosin, tropomyosin, actomyosin)

(ii) Sarcoplasmic proteins (myoalbumin, globulin, enzymes)

(iii) Connective tissue proteins (collagen).

1.1. Collagen

Collagen is a type of biological macromolecule is the most abundant and ubiquitous protein of animal origin, comprising approximately 30 % of total animal protein [4]. It is a natural biomaterial and forms the major component of the extracellular matrix, is vital for the mechanical protection of tissues, organs and physiological regulation of the cellular environment [5]. The name collagen comes from the Greek word “kola”, meaning “glue”, and the suffix –“gen”, denoting “producing” [6]. This refers to the compounds early used in the process of boiling the skin and sinews of horses and other animals to obtain glue.

1.2. Structure of Collagen

The collagen molecule is triple-helical in structure. The structural organization of collagen had been studied by Nobel laureates Pauling [7], Rich and Crick [8] and Yonath [9], followed by significant involvements from prominent scientists namely Ramachandran and Kartha [10], Berman [11], etc. However, the basis of these studies were first laid in 1954 by Ramachandran et al., who researched the concept of the triple-helical arrangement of collagen using kangaroo skin by examining diffraction patterns to estimate a fibre period of 2.86 nm [10]. Collagen is composed of three helical strands inter-twined into one superhelix. It is a heteropolymer that generally consists of two identical chains (α1) and an additional chain that differs slightly in its chemical composition (α2). Normally, collagen is composed of three polypeptide chains of more than 1000 amino acids each. Different polypeptides consisting of all essential amino acids except tryptophan or cysteine and are very low in tyrosine and histidine. Glycine, proline and hydroxyproline are the major amino acids present in collagen at 10-20 folds the concentration found in other proteins. These three amino acids are very important for the formation of the specific triple-helical structure and stabilization of the collagen molecule. The most common motifs in the amino acid sequence of collagen are glycine-proline-X and glycine-X-hydroxyproline, where X is any amino acid other than glycine, proline, or hydroxyproline [12-14]. These three left-handed helices are twisted together into a right-handed coiled-coil, a triple-helix which represent a quaternary structure of collagen, being stabilized by numerous hydrogen bonds and intra-molecular Vander Waal interactions as well as some covalent bonds and further associated with right-handed microfibrils (~40 nm in diameter) and fibrils (100-200 nm in diameter), being further assembled into collagen fibers with unusual strength and stability. Molecules of collagen is about 1.5 nm diameter, 280 nm in length and has a molecular weight of approximately 300 kDa [15-17]. The helical structure of collagen is given in **Fig. 1.1**.

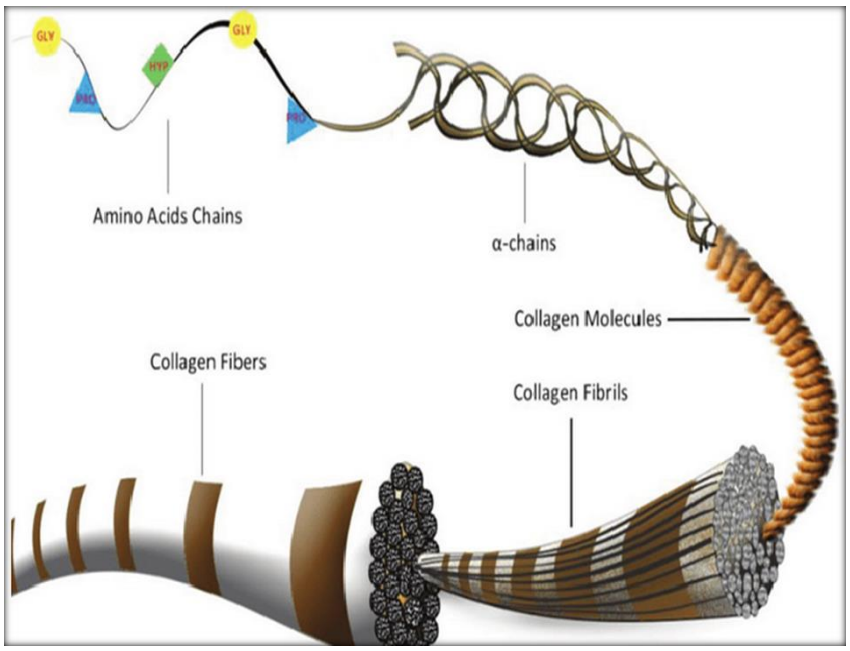

Fig. 1.1. Helical structure of collagen

1.3. Types of Collagen

There are at least 28 different types of collagen so far identified, however, type I to IV constitute 90 % of them. The majority of the collagens can be classified according to the nature of their structure and supramolecular organization [18-20]. A different types of collagen structure is given in **Fig. 1.2**.

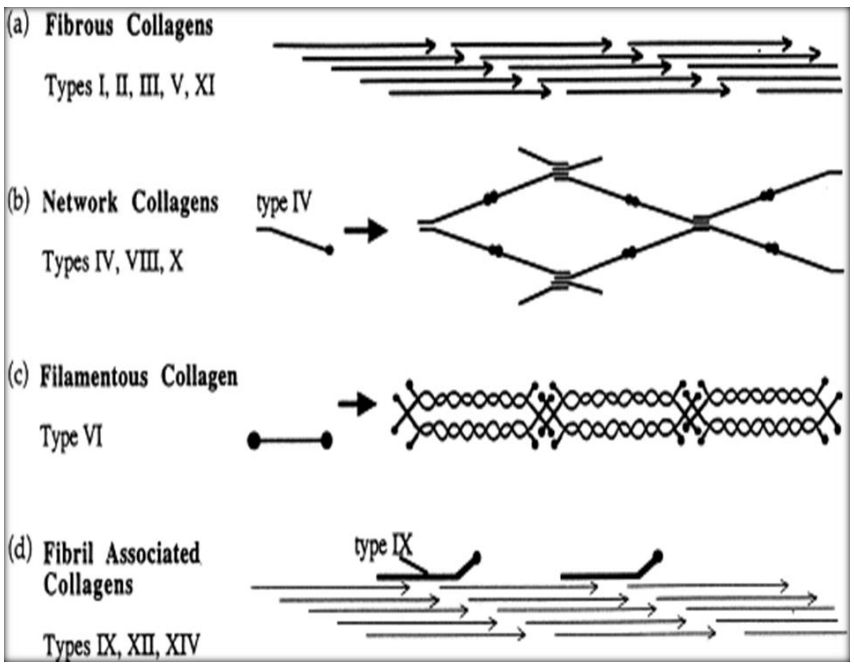

Fig. 1.2. Different types of collagen structure

1.4. Sources of Collagen

The main sources of industrial collagens were those from the skin and bone of pig and cattle. Collagen could be isolated from birds, frogs, equine tendons, etc. But the outbreaks of mad cow disease had resulted in anxieties amongst users of cattle collagen. Besides, the collagen from pig's skin and bone was not allowed to be used in some regions due to religious reasons. Therefore, a variety of alternative sources for collagen had been proposed, including freshwater and marine fishes and different marine animals, such as jumbo squid and jellyfish [21-23]. The bones, skin, fins, scales of fresh or saltwater fishes were mainly used for collagen isolation.

1.5. Benefits of Fish Collagen over Mammalian Collagen

Collagen extracted from fish had several advantages compared with bovine and porcine collagen. It was significantly less cross-linked than mammalian collagen. Therefore, the fish collagen solubility was much higher than bovine and porcine collagen. It allowed to obtain a native form of collagen in the final product by using non-invasive methods while getting collagen from bovine and pig skins required aggressive chemical and/or enzymatic treatment that caused its degradation [24]. Fish collagen had a similar amino acid sequence to that of mammal collagen and it was abundant, cheaper, and did not cause any infectious diseases [25-27]. The denaturation temperature of mammalian collagen was reported to be higher than 30 °C, while most fish collagens denature at a temperature below 30 °C [28]. Higher thermal stability of mammalian collagen could be attributed to the relative abundance of imino acids (hydroxyl proline and proline), leading to higher cross-linked density. The hydroxyproline content was lower in fish collagen due to labile cross-links as compared to mammals [29, 30]. In addition, fish collagens had lower thermal stability than mammalian collagens because fish collagens contained lesser amino acids compared to mammals [31-35].

The reuse of fish processing wastes for biomaterials, such as pharmaceutical microencapsulation, light-sensitive coatings, and food packaging films, might increase the economic value of the fish. Regarding the applications of collagen in numerous fields, it was planned to isolate collagen from two fish varieties and evaluate for their biological and optical potentials.

1.6. STUDY OBJECTIVES

Based on the extensive literature review it was found that collagen could be obtained from different varieties of marine fishes but there is little report available regarding Indian oil sardine (*Sardinella longiceps*) and Indian mackerel (*Rastrelliger kanagurta*). Therefore it was thought worthwhile to isolate collagen from Indian oil sardine and Indian mackerel and to prepare composites for further biological applications.

Based on the literature review conducted it is envisaged that the following objectives could be undertaken.

1. To isolate collagen from *Sardinella longiceps* (Indian oil sardine) and *Rastrelliger kanagurta* (Indian mackerel) and characterize the biochemical properties by analytical and spectroscopic techniques.
2. To fabricate Piscean collagen-based composites materials for wound closure applications.
3. To fabricate nano metal oxide/collagen composites for packaging applications.
4. To study applications of collagen-based functional materials for UV protection and other properties.

SECTION I: ISOLATION AND CHARACTERIZATION OF PISCEAN COLLAGEN

CHAPTER II

ISOLATION OF COLLAGEN FROM INDIAN OIL SARDINE (*Sardinella longiceps*) AND ITS BIOCHEMICAL CHARACTERIZATION

Indian oil sardine (*Sardinella longiceps*) is one of the most important commercial fishes available in Arabian Sea at the west coast of India as shown in **Fig. 2.1.** They belong to the species of ray-finned fish in the genus *Sardinella* and Clupeidae family, found in the northern region of Indian Ocean. The fish was used as bait, for immediate consumption, for canning, parched, seasoning or vaporization and for the diminution into fish nutrition or lipid. The major portion of the sardine is used for human consumption. Indian oil sardines are considered as sustainable seafood. The edible parts of fish sardines are rich in proteins and amino acids, essential for building cardiac muscles and developing the

connective tissues, enzymes and antibodies. These proteins are also used in tissue engineering and regeneration, cosmetics and skincare applications. Fourteen species of sardines were reported in the Indian waters. Out of which, *Sardinella. longiceps* is the major single-species fishery, which contributes about 15 % of the total marine fish production in the country. Therefore, the extraction and purification of ASC from wastes of Indian oil sardine for potential use and as an alternative source for mammalian collagen is envisaged.

Scientific classification:

Kingdom : Animalia
Phylum : Chordata
Class : Actinopterygii
Order : Clupeiformes
Family : Clupeidae
Genus : *Sardinella*
Species : *longiceps*

Fig. 2.1. Indian oil sardine (*Sardinella longiceps*)

2.1. MATERIALS AND METHODS

2.1.1. Collection of samples

Indian oil sardines were collected from the fish market at Mangalore. It was packed in a polyethylene bag, kept in ice and transported to the laboratory. The bone, skin, fins and head portions of the fish were removed and washed with cold distilled water, stored at -20°C till used.

2.1.1.1. Isolation of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from the bone, skin, fins and head of Indian oil sardine (*Sardinella longiceps*)

(i) Isolation of acid-soluble collagen (ASC)

The acid-soluble collagen was isolated from the different parts of Indian oil sardine *(Sardinella longiceps)* using the method reported by Nagai and Suzuki [36] with a slight modification. The fishbone, skin, fins and head were removed and weighed. The samples were washed with cold distilled water for a few minutes and ground. Collagen was extracted with 0.1 M NaOH for 48 h at a sample/alkali solution ratio of 1:10 (w/v). The process was repeated every 5 h to ensure complete removal of non-collagenous matter. The residue was washed with cold distilled water until the pH value was close to neutral. The bone and fin samples were found to contain more calcium so those were decalcified with 0.5 M disodium EDTA solution (pH 7.5) for 5 days. The fat was removed by adding 10 % butyl alcohol at a sample/alcohol ratio of 1:10 (w/v) and stirring overnight, after that, the samples were washed with cold distilled water. The residue was extracted with 0.5 M acetic acid at a sample/acid ratio of 1:4 (w/v) for 3 days. The insoluble matter was re-extracted with the same solution at a sample/acid ratio of 1:2.5 (w/v) for 2 days. Each extract was filtered by cotton cloth and then centrifuged at 10000 rpm for 30 minutes. The supernatant was salted out by adding NaCl to a final concentration of 2.5 M in the presence of 0.05 M Tris-HCl, pH 7.0, stirred well and kept overnight to precipitate the collagen. The resultant precipitate was collected by centrifuging at 10000 rpm for 30 minutes. Pellet was dissolved in 0.5 M acetic acid and dialyzed against 0.1 M acetic acid and distilled water respectively. Amicon Ultra centrifugal filters (Millipore) 100 kDa cut-off filters were used for concentrating the crude collagen. The dialyzed collagen solution (10 mL) was taken in an Amicon centrifugal tube and it was centrifuged at 5000 rpm for 15 min at 4 °C. The retentate was collected and lyophilized. It was labelled as

concentrated acid-soluble collagen (ASC) and stored at 4 °C.

(ii) Isolation of pepsin-soluble collagen (PSC)

The residue obtained from the acetic acid extraction were suspended in 0.5 M acetic acid and digested with 0.5 % (w/v) pepsin 1:10000 for 72 h and entrifuged at 10000 rpm for 30 minutes. The supernatant was salted out and the remaining extraction steps of PSC were same as the extraction of ASC.

Collagen yield (dry basis) was determined by the following formula = $$\frac{(\text{Weight of final collagen sample in g})}{(\text{weight of sample in g})} X100$$

The schematic representation for the isolation of acid-soluble and pepsin-soluble collagen is shown in **Fig. 2.2.**

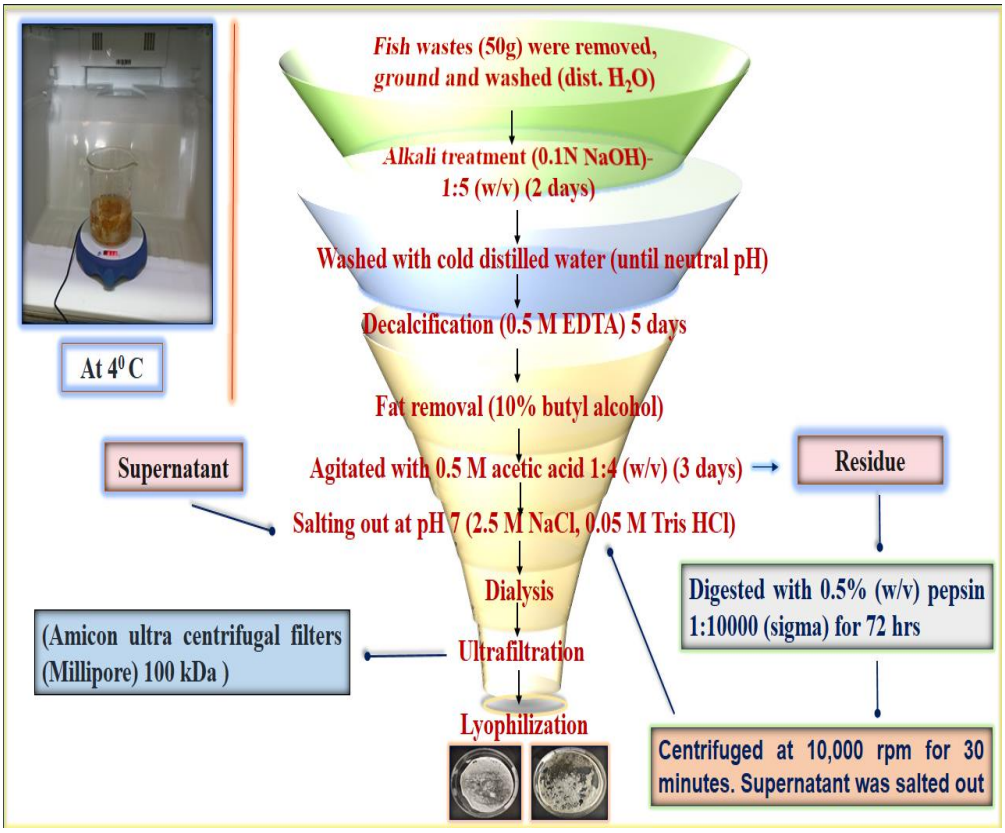
Fish wastes (50g) were removed, ground and washed (dist. H_2O)
Alkali treatment (0.1N NaOH)- 1:5 (w/v) (2 days)
Washed with cold distilled water (until neutral pH)
Decalcification (0.5 M EDTA) 5 days
Fat removal (10% butyl alcohol)
Agitated with 0.5 M acetic acid 1:4 (w/v) (3 days)
Residue
Supernatant
Salting out at pH 7 (2.5 M NaCl, 0.05 M Tris HCl)
Dialysis
Digested with 0.5% (w/v) pepsin 1:10000 (sigma) for 72 hrs
(Amicon ultra centrifugal filters (Millipore) 100 kDa)
Ultrafiltration
Lyophilization
Centrifuged at 10,000 rpm for 30 minutes. Supernatant was salted out
At 4^0 C

Fig. 2.2. The schematic diagram for the isolation of acid-soluble and pepsin-soluble collagen from Indian oil sardine.

2.2. RESULTS AND DISCUSSION

The isolated ASC and PSC samples were found to be an odorless and whitish fiber-like appearance. The yield of ASC from bone, skin, fins and head was found to be 1.8 % (± 0.12 SD), 2.5 % (± 0.10 SD), 1.2 % (± 0.11 SD) and 2.1 % (± 0.12 SD) respectively. The yield of PSC from bone, skin, fins and head was found to be 0.65 % (± 0.02 SD), 0.81 % (± 0.01 SD), 0.21 % (± 0.03 SD) and 0.70 % (± 0.02 SD) respectively, based on the lyophilized dry weight. The acid-soluble skin collagen showed a higher yield compared to that of pepsin-soluble collagen. The SDS-PAGE characterization revealed that the collagen isolated is of type-I with that of reference type-I ASC of calf-skin collagen. Upon amino acid analysis the skin collagen of Indian oil sardine had high serine and glycine content with a notable absence of tryptophan residue. The scanning electron microscopy (SEM) analysis of the surface exhibited densely packed collagen fibers that have similar morphology with that of reference ASC of calf-skin collagen. The purity of ASC was identified by UV-Vis spectroscopy where the isolated collagen exhibited λ_{max} at 225 nm. The absence of an absorption band at 280 nm confirmed the absence of aromatic amino acids in the isolated protein collagen. In the FTIR spectrum, five amide bands were identified as major bands. The maximum solubility of ASC was observed at pH 4 and 4 % NaCl could be due to the "salting in" effect. A sharp decrease was observed above the pH 4 and NaCl concentration above 4 %.

CHAPTER III

ISOLATION OF COLLAGEN FROM INDIAN MACKEREL (*Rastrelliger kanagurta*) AND ITS BIOCHEMICAL CHARACTERIZATION

Indian mackerel (*Rastrelliger kanagurta*) is a species of mackerel in the scombrid family (family *scombridae*) of order *perciforms.* It is commonly found in the Indian and West Pacific oceans, and their surrounding seas as shown in **Fig. 3.1**. It is an important meals fish and is commonly utilized in south and south-east Asian cuisine. Indian mackerel is one of the highly recommended oily fish for a healthy diet. It is rich in essential oils, vitamins and minerals. Omega-3 and Omega-6 fatty acids occur in excessive quantities in this fish. The fish also contains protein and the antioxidant coenzyme Q10. The fish has several health benefits. The health benefit of mackerel is very effective in regulating

hormonal levels and also improves the elasticity of blood vessels and capillaries. It is beneficial in lowering bad cholesterol levels as it has the property of reducing blood coagulation. Indian mackerel fish even lowers high blood pressure levels. It prevents heart-related disorders, improves organ functions that are weakened by illnesses, strengthens the immune system and regulates metabolism. Because of these purposes, the collagen extracted from Indian mackerel is used as an opportunity supply of collagen to be used in diverse applications.

Scientific classification

Kingdom : Animalia

Phylum : Chordata

Class : Actinopterygii

Order : Perciformes

Family : Scombridae

Genus : *Rastrelliger*

Species : *kanagurta*

Fig 3.1. Indian mackerel (*Rastrelliger kanagurta*)

3.1. MATERIALS AND METHODS

The materials, methods of preparations and instruments used for the study have been already described in chapter 2. The similar techniques were used.

3.1.1. Isolation of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from the bone, skin, fins and head of Indian mackerel (*Rastrelliger kanagurta*)

The isolation of acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were carried out according to the methods described in chapter 2.

3.2. RESULTS AND DISCUSSION

The acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) were isolated from the fish bone, skin, fins and head of Indian mackerel (*Rastrelliger kanagurta*) were found to be odorless and whitish fiber-like appearance. The yield of ASC from fish bone, skin, fins and head was found to be 1.92 % (± 0.12 SD), 3.57 % (± 0.10 SD), 1.66 % (± 0.12 SD) and 3.14 % (± 0.11 SD) respectively. The yield of PSC from fish bone, skin, fins and head was found to be 0.78 % (± 0.03 SD), 1.17 % (± 0.01 SD), 0.54 % (± 0.04 SD) and 0.81 % (± 0.03 SD) respectively, on the basis of lyophilized dry weight. The acid-soluble skin collagen showed a higher yield compared to that of pepsin-soluble collagen. The SDS-PAGE characterization revealed that the collagen isolated is of the type-I with that of reference type-I ASC of calf-skin collagen. Upon amino acid analysis the skin collagen of Indian mackerel had high serine and glycine content with notable absence of tryptophan residue. The SEM analysis of the surface exhibited densely packed collagen fibers which has similar morphology with that of reference ASC of calf-skin collagen. The purity of ASC was identified by UV-Vis spectroscopy where the isolated collagen exhibited λ_{max} at 225 nm. The absence of an absorption band at 280 nm confirmed the absence of aromatic amino acids in the isolated protein collagen. In the FTIR spectrum, five amide bands were identified as major peaks. The maximum solubility of ASC was observed at pH 4 and 4 % NaCl could be due to the “salting in” effect. A sharp decrease was observed above the pH 4 and NaCl concentration above 4 %.

SECTION II: BIOLOGICAL APPLICATIONS OF PISCEAN COLLAGEN

CHAPTER IV

STUDIES ON WOUND CLOSURE APPLICATIONS OF ACID-SOLUBLE COLLAGEN/NANO METAL OXIDE COMPOSITES

4.1. A Study on the Synergistic Effect of Bioactive Compounds from Papaya Leaf and Collagen Isolated from Indian Mackerel on *in vitro* Wound Closure Capacity

Wound healing is an essential biological phenomenon that involves tissue repair and regeneration. *Carica papaya* belongs to the Caricaceae family, possesses antimicrobial, antioxidant and anti-inflammatory properties and also exhibits excellent wound healing activity. Major phytochemicals such as alkaloids, flavonoids, glycosides, phenolics, and saponins, etc., have been isolated from *C. papaya* leaves. Important phenolic compounds found in papaya leaf extracts include caffeic acid, p-coumaric acid, quercetin, protocatechuic acid, 5, 7-dimethoxy coumarin, kaempferol and chlorogenic acid, which act as free radical terminators [37, 38]. Enormous reports are available associated with the wound healing capabilities of leaf extracts. However, there are limited reports available on the synergistic effect of individual leaf constituents along with extracellular matrix (ECM) proteins such as collagen. Therefore, in the present study, specific leaf constituents along with collagen gel were evaluated for their wound healing activity.

4.1.1. MATERIALS AND METHODS

4.1.1.1. Gel formulations for *in vitro* wound closure activity using collagen with glycerol and papaya leaf constituents

In the present study, the synergistic effects of leaf constituents were evaluated along with extracted ASC from the skin of Indian mackerel. The collagen gels were prepared along with the major constituents found in papaya leaf, including caffeic acid, coumaric acid, quercetin and oleic acid. The tests were carried out in one-to-one combinations as well as in mixed combinations. A series of gel samples were prepared with different constituents, as shown in **Table 4.1.1.**

Table 4.1.1. Gel formulations tested for wound closure activity

Test compound	Composition of gel
Sample 1 (S1)	Collagen (5% w/v)+glycerol (2:1)
Sample 2 (S2)	Collagen+glycerol+0.25 mg caffeic acid
Sample 3 (S3)	Collagen+glycerol+0.34 mg coumaric acid
Sample 4 (S4)	Collagen+glycerol+0.04 mg quercetin
Sample 5 (S5)	Collagen+glycerol+0.25 mg caffeic acid+0.34 mg coumaric acid+0.04 mg quercetin
Sample 6 (S6)	Collagen+glycerol+0.25 mg caffeic acid+0.34 mg coumaric acid+0.04 mg quercetin+oleic acid

4.1.2. RESULTS AND DISCUSSION

The present study investigated the effect of collagen gel along with different papaya leaf constituents on *in vitro* wound healing assays using the NIH3T3 cell line. The FTIR study confirmed the strong intermolecular hydrogen bonding between collagen and leaf constituents by shifting of absorption bands to lower positions. Results of *in vitro* cytotoxicity tests showed that prepared collagen gel did not induce a significant cytotoxic effect and could be utilized as a safe material for preparing biocompatible collagen-based gels. The most effective migration was observed in sample 4 (collagen+glycerol+quercetin) at 25 $\mu g \cdot mL^{-1}$ concentration, with 100 % migration of cells and resulting in complete wound closure **(Fig. 4.1.1).** As a result, extracted collagen gel could be used as an effective biomaterial for wound dressing.

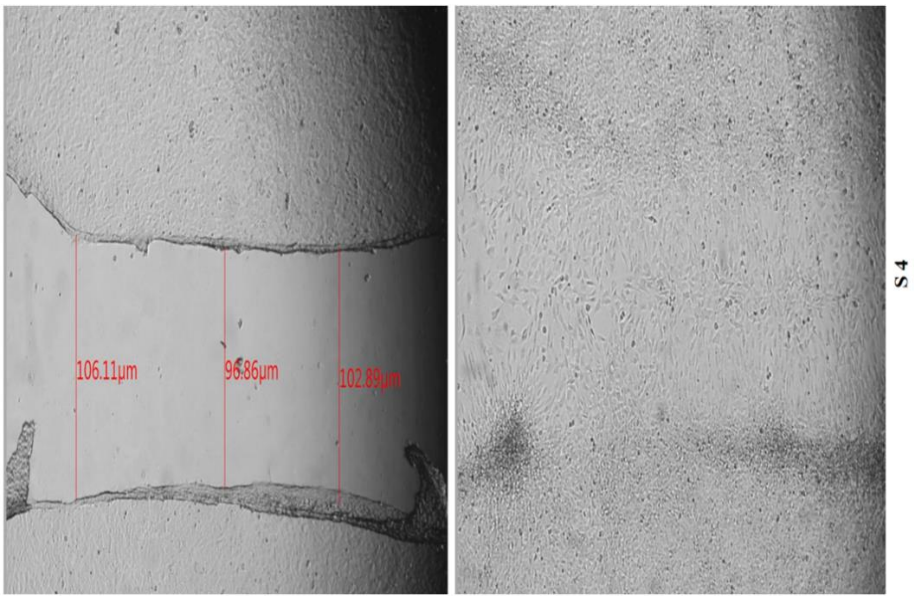

Fig. 4.1.1. Photographs of scratch assays on NIH3T3 cells at zero and 24 h under 40 X magnification (Sample S4).

4.2. Fabrication of Electrospun collagen/TiO_2 Nanocomposite for *in vitro* Wound Closure Activity

Electrospinning technology is one of the most popular manufacturing process for the preparation of porous nanofibers using an electrostatically driven jet of a polymer solution. It is also a fast and simple polymer processing technique [39]. The fibers produced by electrospinning technique exhibit numerous advantages such as small diameter generally in the range of several nanometers to a few micrometers [40], a high surface area to volume ratio which would accelerate the solubility of a drug in the aqueous medium and enhance the efficiency of the drug, high tunable porosity and handling nanofiber composition in order to achieve desired properties and function [39]. Wound exposure often causes bacterial infections and inflammation, which further affects wound healing. Therefore, if anti-inflammatory and antibacterial drugs can be incorporated into wound dressings, they would be of great benefit for skin repair.

4.2.1. MATERIALS AND METHODS

4.2.1.1. The fabrication of [(PVA/collagen)-(TiO_2-NPs)] electro-spun nanofibers

The acid-soluble collagen was isolated from the skin of Indian mackerel (*Rastrelliger kanagurta*) was used for the preparation of PVA/collagen nanofiber. PVA acts as a base matrix for the preparation of PVA/collagen nanofiber. Blends of the PVA (10 %) and

collagen (2 %) doped with TiO_2-NPs have been prepared in the form of nanofiber by the electrospinning technique. The fabrication of various electrospun nanofibers with different compositions of host material and dopant was done in order to investigate the maximum amount host polymer and dopant could be accommodated without tempering the physical structure and morphology of the host polymer. Prequel to nanofibers formations, solutions containing a varied amount of polymer blend and or doped polymer blend was prepared to achieve a desired and electrospinnable viscosity. Different concentrations of TiO_2-NPs suspension (0.25 %, 0.5 %, 1 %) (w/v) were added to PVA (10 %) / collagen (2 %) blend solution and it was stirred at 4 °C, 350 rpm for 12 h on a magnetic stirrer. Each of the prepared solutions (5 mL) was fed into a plastic syringe (diameter 20 mm) and the latter was adjusted to 15 cm away from drum collector. The solution was treated with 20 kV and a drum collector speed of 300 rpm. The flow rate was adjusted to 0.5 mL/h. The obtained nanofibers were collected in a polyethene bag. The schematic representation for the preparation of different concentrations of TiO_2-NPs doped PVA/collagen nanofiber mats are shown in **Fig. 4.2.1.**

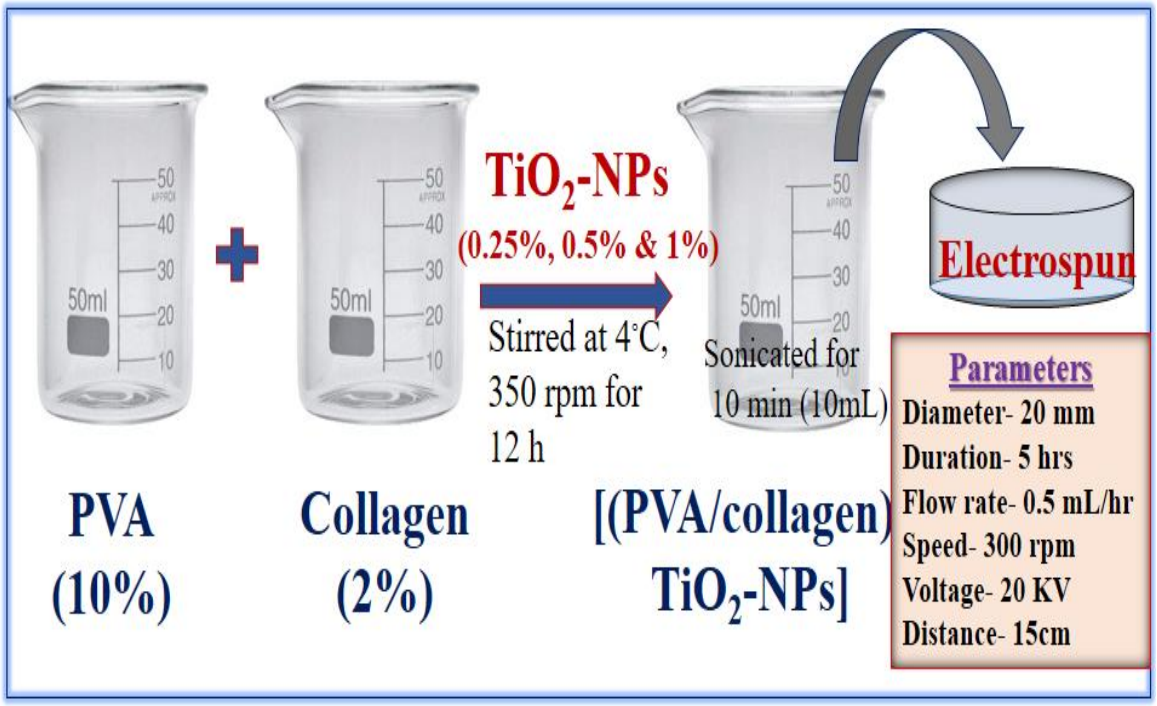

Fig. 4.2.1. The schematic representation of the preparation of different concentrations of TiO_2-NPs doped PVA/collagen nanofibers

4.2.2. RESULTS AND DISCUSSION

This study investigated the effect of PVA/collagen nanofiber along with different concentrations of TiO_2-NPs on *in vitro* wound healing assays using the NIH3T3 cell line. The transformation of the films into crystallinity and the presence of the Ti elements were confirmed by SEM and EDAX. The results of TGA revealed that the addition of nanoparticles influenced the thermal stability of the PVA/collagen blended nanofibers. Further, the powder X-ray diffraction study revealed the amorphous nature of PVA/collagen blended film which was modified into a crystalline structure after the addition of TiO_2 nanoparticles. From the FTIR data, it could be confirmed that there was significant interaction among the PVA/collagen/ TiO_2-NPs. The weak interactions like H-bonding was evident by the shift of the absorption band of hydroxyl groups into the lower frequency region. The addition of TiO_2 nanoparticles leads to an increase in the stiffness and mechanical resistance of the nanocomposite films; which showed an increase in their tensile strength evidenced by an increase in Young's modulus. These test compounds were screened by *in vitro* scratch wound assays using the NIH3T3 cell line. Among all the test samples, PVA/collagen with TiO_2-NPs (1 %) was the most effective in enhancing wound closing, with 65.45 % migration of fibroblasts **(Fig. 4.2.2)**. As a result, collagen extracted from the skin of Indian mackerel can be used as an effective biomaterial for wound dressing. The marine material can be used as an alternative to bovine or human collagen in selected biomedical applications.

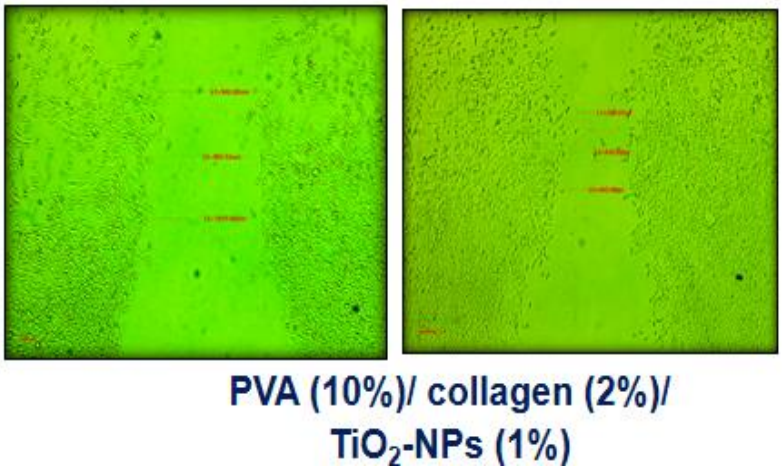

Fig. 4.2.2. Photographs of scratch assays on NIH3T3 cells at zero and 24 h under 40 X magnification

CHAPTER V

FABRICATION OF ENVIRONMENTALLY COMPATIBLE BIOPOLYMER FILMS OF PULLULAN/PISCEAN COLLAGEN/ZnO NANOCOMPOSITE AND THEIR ANTIMICROBIAL ACTIVITY

Biopolymers were used as edible film packaging or coating materials in the food industry as they increase sustainability while maintaining food product quality and further contribute to control the foodborne pathogens [41-46]. Hence, it is a challenge to prepare new polymer composite films for the production of packaging or coating material from biodegradable natural renewable resources and also to solve the waste disposal complications. Pullulan might be considered as the foremost matrix of such a film. The biopolymer's performance could be further enhanced by fabricating with natural fiber or protein and were considered as composites.

5.1. MATERIALS AND METHODS

5.1.1. The preparation of [(pullulan/collagen)-(ZnO-NPs)] nanocomposite film

The acid-soluble collagen was isolated from the skin of Indian mackerel (*Rastrelliger kanagurta*) was used for the preparation of pullulan/collagen blend film. Pullulan acts as a base matrix for the preparation of a pullulan/collagen blend film. Blends of the pullulan and collagen doped with ZnO-NPs have been prepared in the form of films by using the solution casting method. Different concentrations of ZnO-NPs suspension (0.25 %, 0.5 % and 1 %) (w/v) were added to pullulan (2 %) / collagen (2 %) blend solution and it was stirred at 4 °C, 350 rpm for 12 h on a magnetic stirrer. After the completion of the reaction, the doped solution (30 mL) was sonicated for 10 minutes and it was uniformly poured onto the Petri plate as thin film and dried at 40 °C for 24 h. The films were cooled at room temperature, slowly peeled off from the Petri plate and stored in a polythene bag. The schematic representation for the preparation of different concentrations of ZnO-NPs doped pullulan/collagen nanocomposite films as shown in **Fig. 5.1.**

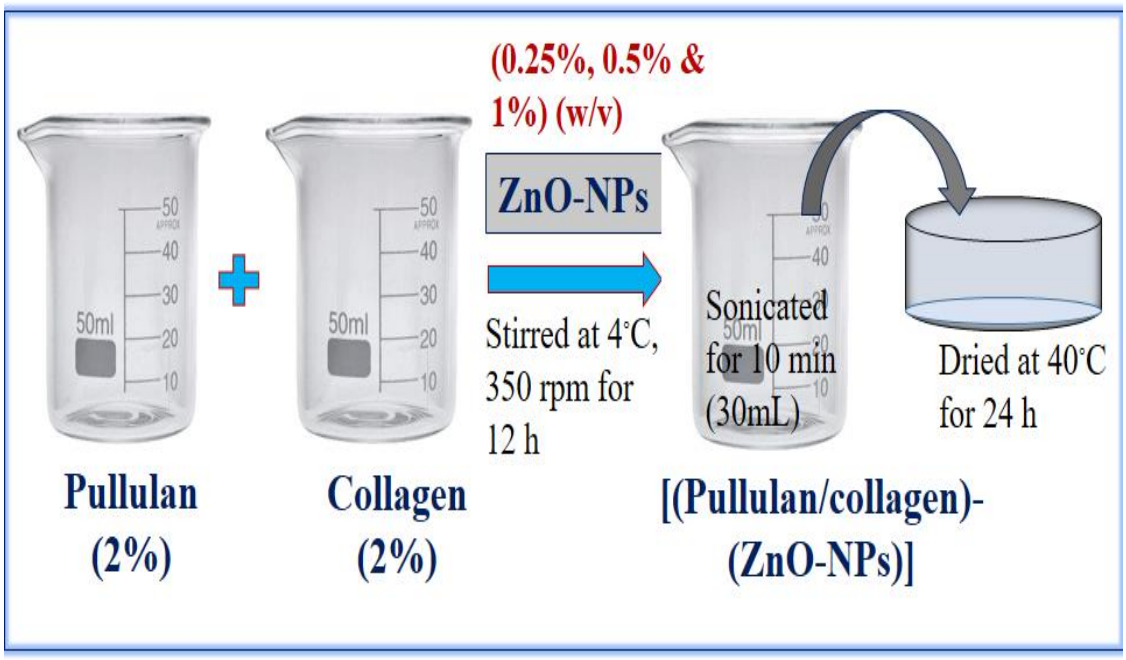

Fig. 5.1. The schematic representation for the preparation of different concentrations ZnO-NPs doped pullulan/collagen nanocomposite films

5.2. RESULTS AND DISCUSSION

The present chapter highlights a study on the preparation and characterization of different concentrations of ZnO-NPs doped pullulan/collagen film and evaluation of their antimicrobial property for edible packaging film. Polysaccharide pullulan acts as a base matrix for the fabrication of pullulan/collagen blend film with dopant ZnO nanoparticles. The transformation of the films into crystallinity and the presence of the Zn elements were confirmed by SEM and EDAX. The results of TGA revealed that the addition of nanoparticles influenced the thermal stability of the pullulan/collagen blended films. Further, the powder X-ray diffraction study revealed the amorphous nature of pullulan/collagen blended film which was modified into a crystalline structure after the addition of ZnO nanoparticles. From the FTIR data, it could be confirmed that there was significant interaction among the pullulan/collagen/ZnO-NPs. The weak interactions like H-bonding was evident by the shift of the absorption band of hydroxyl groups into the lower frequency region. The addition of ZnO nanoparticles lead to an increase in the stiffness and mechanical resistance of the nanocomposite films; which showed an increase in their tensile strength evidenced by an increase in Young's modulus. The fabricated films exhibited antifungal activity against *Aspergillus niger* with a maximum

zone of inhibition of 18 mm for pullulan/collagen/ZnO-NPs (0.5 %) **(Fig. 5.2).** However, the nanocomposite films easily dissolved in an aqueous medium, indicating their possible use as edible packaging films in the food industry.

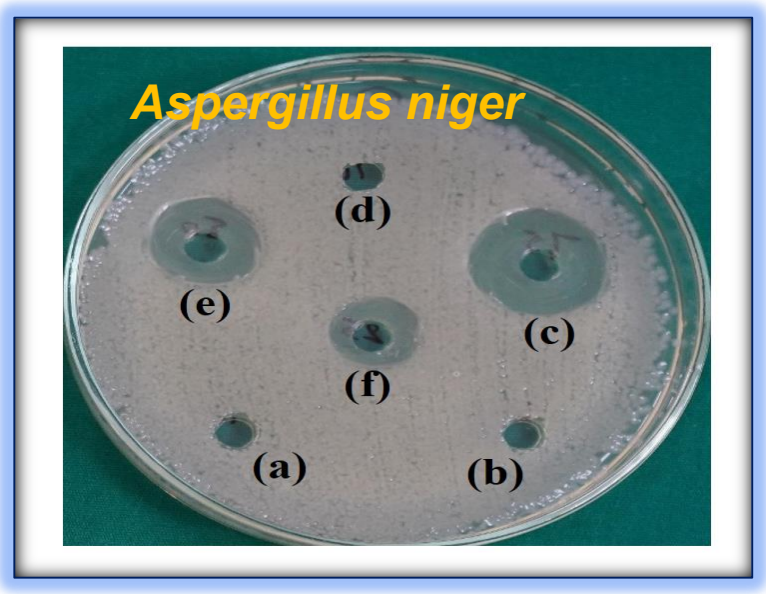

Fig. 5.2. Antifungal (*Aspergillus niger*) activity images of pullulan/collagen blend films doped with different concentrations of ZnO nanoparticles (a) Pullulan (2%), (b)Pullulan(2%)/collagen(2%), (c)Standard (Fluconazole), (d) Pullulan/collagen/ZnO-NPs (0.25%), (e) Pullulan/collagen/ZnO-NPs (0.5%), (f) Pullulan/collagen/ZnO-NPs (1%)

CHAPTER VI

DETERMINATION OF SUN PROTECTION FACTOR (SPF), ULTRA PROTECTION FACTOR (UPF) AND FLUORESCENCE QUENCHING PROPERTY OF ACID-SOLUBLE COLLAGEN/ORGANIC DOPANT COMPOSITE

6.1. A Study on UV- B Radiation Filtering Films Prepared by Piscean Collagen and 2-(4-tert-butylphenyl)-5-4-biphenyl)-1,3,4-oxadiazole (PBD)

Long exposure to UV radiation may damage living organisms because UV photons have a sufficient amount of energy to destroy chemical bonds. UV radiations induce structural and biochemical changes by initiating photochemical reactions [47]. Type-I collagen is considered as an abundant protein found in the extracellular matrix and is responsible for

skin strength and elasticity. Its degradation leads to wrinkles that accompany aging. Continuous UV irradiation from the sun reduces the production of type-I collagen which is the major structural protein found in human skin, the reduction of type-I collagen causes premature skin aging which is associated with skin carcinoma [48]. Therefore, many UV blocking materials were developed to protect against UV radiation. The UV blocking materials have properties to filter the UV radiation in the wavelength range of 200-400 nm. This can be subdivided into three regions such as UV-C (200-290), UV-B (290-320) and UV-A (320-400) [49, 50]. The UVB is primarily associated with erythema, sunburn, immunosuppression and photocarcinogenesis [51]. There are two types of UV blockers namely, inorganic and organic. Organic UV blockers predominantly absorb UV rays, and therefore known as UV absorbers [52].

6.1.1. MATERIALS AND METHODS

6.1.1.1. The preparation of PVA/PBD/collagen composite film

The PVA/PBD/collagen composite films have been prepared in the form of films by solution casting method. Different concentration (2 %, 4 % and 6 %) of collagen was added to PVA (2 %) solution and it was stirred at 4 ˚C, 350 rpm for 12 h on a magnetic stirrer. The PBD (2 %) was added dropwise to the PVA/collagen blend solution. After the completion of reaction, the doped solution was uniformly poured into the Petri plate as thin film and dried at 40 ˚C for 24 h. The films were cooled to room temperature, slowly peeled off from the petri dish and stored in a polyethene bag. The schematic representation for the preparation of different concentrations of collagen doped PVA/PBD composite films as shown in **Fig. 6.1.1.**

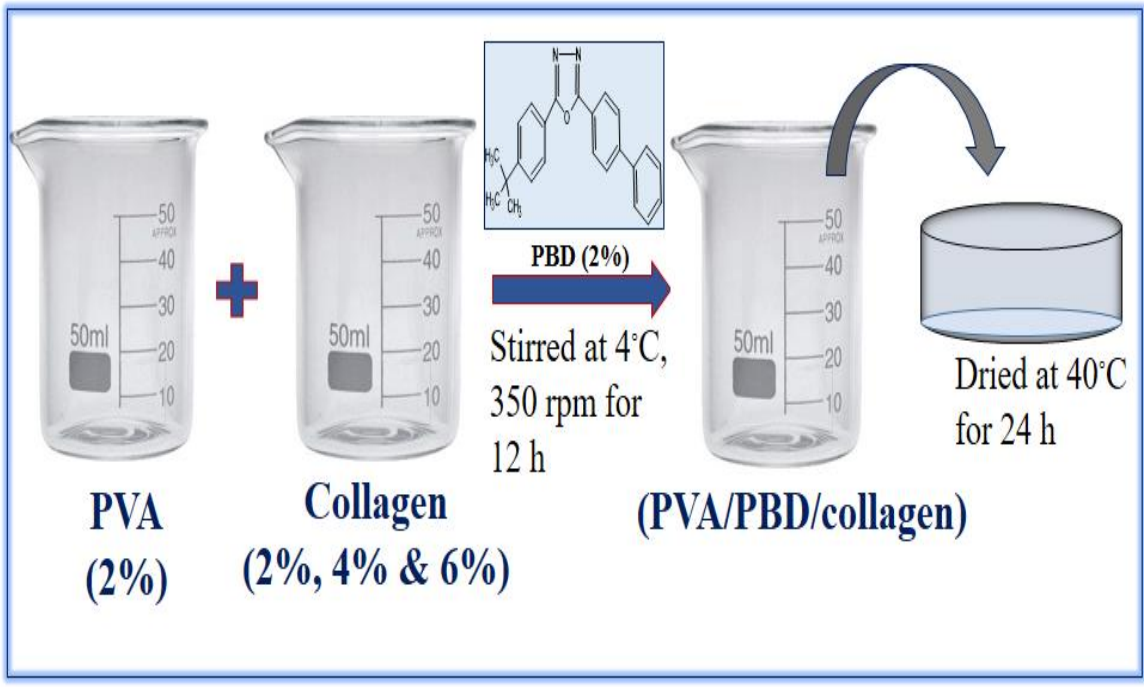

Fig. 6.1.1. The schematic representation of the preparation of different concentrations of collagen doped PVA/PBD composite films

6.1.2. RESULTS AND DISCUSSION

The present section reports a study on the preparation of a new type of selective UV-filtering material PVA/PBD/collagen was prepared by solvent casting method. The confirmatory characterization of fabricated films were carried out by FESEM, TGA and FTIR respectively. The UPF factor was calculated *in vitro* by UV-VIS spectroscopy. The From SEM analysis, increased concentration of collagen resulted in uniform distribution of PVA/PBD with collagen. The TGA result revealed that the thermal stability of the composite has been improved by incorporating a higher concentration of collagen into PVA/PBD blended films. The FTIR analysis showed that there was a strong hydrogen bond formed between PVA/PBD/collagen films. The addition of different concentrations of collagen led to an increase of the stiffness and mechanical resistance of the composite films were evidenced by an increase in Young's modulus and a decrease in elongation at break. The density of the cross-linked films were increased when increased concentrations of collagen in the matrix. From UV analysis, when the addition of PBD to collagen shifted its λ_{max} to 307 nm in the UV-B (290-320 nm) (**Fig. 6.1.2).** The composite film (PVA/PBD/collagen (6 %)) showed a higher SPF (23.60) and UPF (52.38) value when compared to the pure PVA and PVA/PBD/collagen blend film.

Hence in this study, it was established that PVA/PBD/collagen/ (6 %) composite films provide an excellent solution for UV-B filtering applications and could be used for UV blocking additives for fabrics.

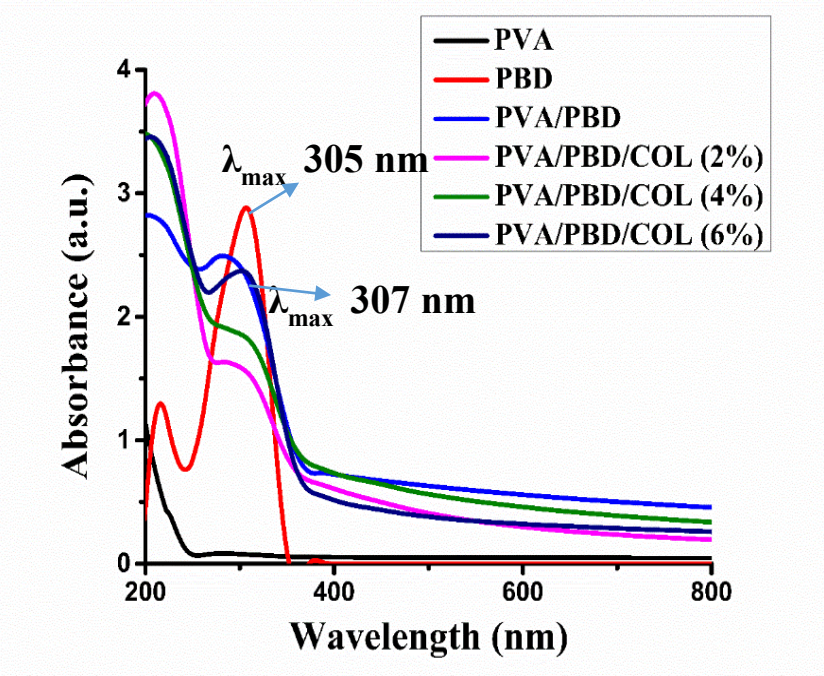

Fig.6.1.2. UV-Visible spectra of PVA/PBD films doped with different concentrations of collagen.

6.2. *In vitro* Determination of UV-A/UV-B Radiation Protection Capabilities of Fabricated Films by Piscean Collagen and Bischalcone derivative (B1)

6.2.1. MATERIALS AND METHODS

6.2.1.1. The fabrication of the PVA/collagen/B1 composite films

The isolated acid-soluble collagen from the skin of Indian oil sardine (*Sardinella longiceps*) was taken for the study. The PVA acts as a base matrix for the preparation of PVA/collagen blend films doped with different concentrations of B1 by the solution casting method. Different concentrations of B1 (0.25 %, 0.5 %, 1 %, 2 % and 4 %) (w/v) were added to PVA (2 %)/collagen (2 %) blend solution and it was stirred at 4 °C (350 rpm) for 12 h on a magnetic stirrer. After the completion of the reaction, the doped solution (30 mL) was sonicated for 5 minutes and it was uniformly poured onto the Petri plate as thin film and dried at 40 °C for 24 h. The films were cooled to room temperature, slowly peeled off from the Petri plate and stored in a polyethylene bag **(Fig. 6.2.1)**.

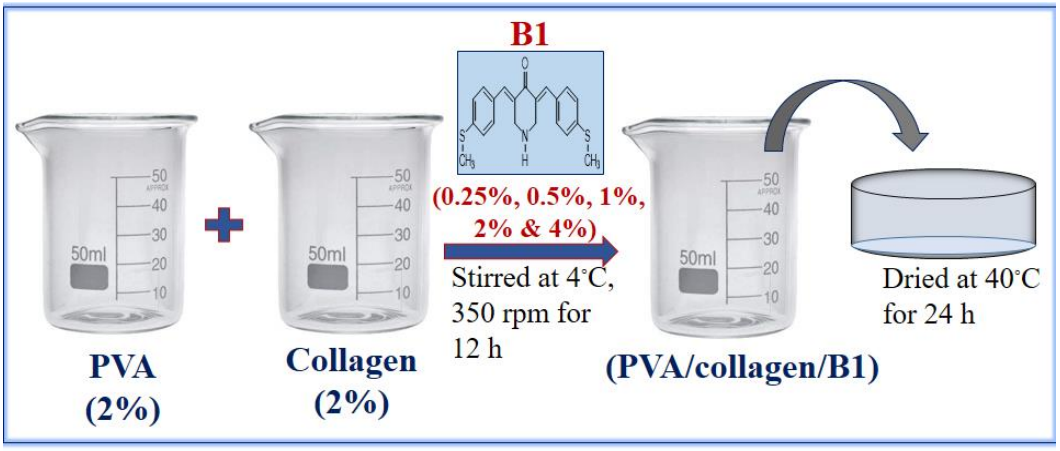

Fig. 6.2.1. The schematic representation for the preparation of different concentrations B1 doped PVA/collagen composite films

6.2.2. RESULTS AND DISCUSSION

The present section reports the development of distinct UV-A and UV-B radiation filtering materials through the introduction of a heterocyclic bischalcone derivative [(3*Z*, 5*E*)-3,5-bis{[4-(methylsulfanyl)phenyl]methylidene}piperidine-4-one] (B1) into the matrix of PVA/Piscean collagen blend films (1:1) prepared through solution casting method and characterized. The dopant concentration varied from 0.25 – 4 %. The SEM images showed a rough surface due to the uniform dispersion of dopant B1. Further, the results of TGA revealed that the addition of B1 increased thermal stability also. From the FTIR data, it could be confirmed that there was significant interaction among the PVA/collagen/B1. The addition of different concentrations of B1 altered mechanical strength with a proportional increase in Young's modulus (146 MPa -317 MPa), tensile strength (23.3 MPa to 39.21 MPa) and decrease in its elongation @ break (158.8 % to 105.2 %). Dopant concentration-dependent increase in density was observed in films (31 to 162 mg/cm^3). The bathochromic shift from 370 nm to 390 nm for λ_{max} as well as hyperchromism was evidenced with an increase in the concentration of B1 **(Fig. 6.2.2).** On determining UV filtering ability for all the prepared films, the one with 4 % dopant showed higher SPF (27.532) and UPF (58.23) values. In addition, the degradation of supercoiled PBR322 DNA on UV irradiation was effectively inhibited by these films with dopant concentration of 0.5-4.0 % (**Fig. 6.2.3).** The inferences of the experiments would indicate the use of these water insoluble films as UV blocking potential materials with merit of SPF and UPF characteristics.

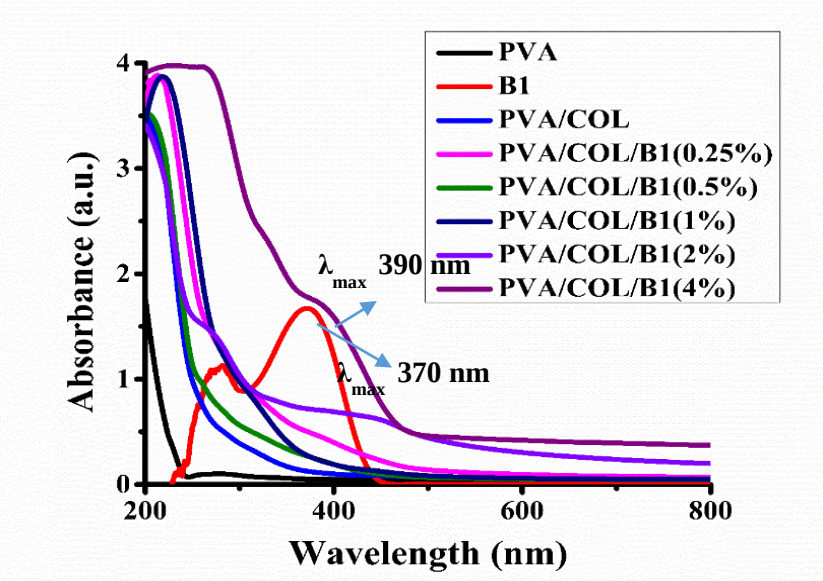

Fig. 6.2.2. UV spectra analysis of PVA/collagen blend films doped with different concentrations of B1 (a) PVA(2%), (b) PVA(2%)/collagen(2%), (c) PVA/collagen/B1 (0.25 %), (d) PVA/collagen/B1 (0.5 %), (e)PVA/collagen/B1 (1 %), (f) PVA/collagen/B1 (2 %), (g) PVA/collagen/B1 (4 %).

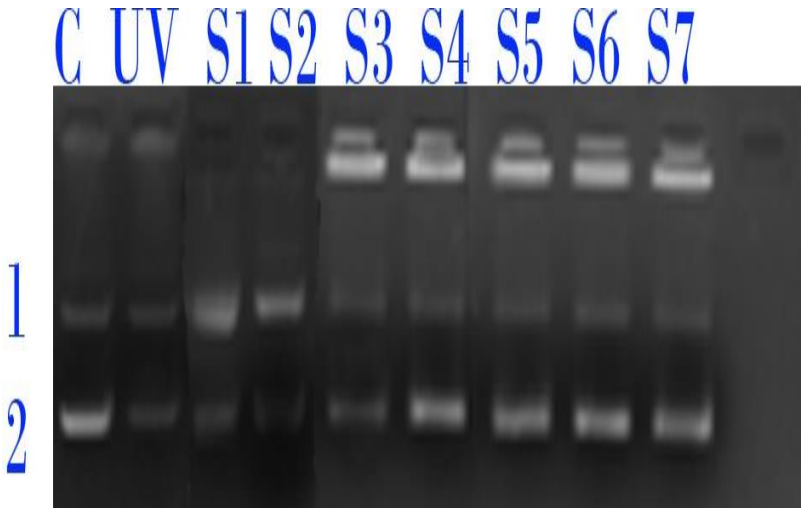

Fig. 6.2.3. The DNA protection study of PVA/collagen blend film doped with different concentrations of B1. Lane 1 (C) shows control PBR322 DNA without photo-irradiation Lane 2 (UV) shows control PBR322 DNA subjected to photo-irradiation (S1) PVA(2%), (S2) PVA(2%)/collagen(2%), (S3) PVA/collagen/B1 (0.25 %), (S4) PVA/collagen/B1 (0.5 %), (S5) PVA/collagen/B1 (1 %), (S6) PVA/collagen/B1 (2 %), (S7) PVA/collagen/B1 (4 %).

6.3. A Study on Fluorescence Quenching Property of Collagen Isolated from Indian oil sardine by Photoluminescence Method

Textile wastewater is known to be a complicated waste to process, due to the presence of both cationic and anionic charges. The use of natural polymers (plant or animal sources) is a promising method for treating wastewater to removing dyes, due to the chemical structure and the composition of the polymers, such as the presence of many functional groups, such as the carboxyl, hydroxyl, phosphate, amine functional groups, which can bind to the cationic charges on the dye molecules by using electrostatic force which contributes effectively towards the removal of dyes from the wastewater. In addition, natural polymers are non-toxic, low-cost, renewable, biodegradable, and biocompatible [53-55].

6.3.1. MATERIALS AND METHODS

6.3.1.1. The preparation of the Collagen/Rhodamine B (RB) solution

Different concentrations (0.25 %, 0.5 %, 1 % and 2 %) of acid-soluble skin collagen prepared in acetic acid (17.4 N) was added to Rhodamine B solution (1 %) in water and it was stirred at 4 °C, 350 rpm for 12 h on a magnetic stirrer. The schematic representation for the preparation of different concentrations of collagen/Rhodamine B solution as indicated in the **Fig. 6.3.1.**

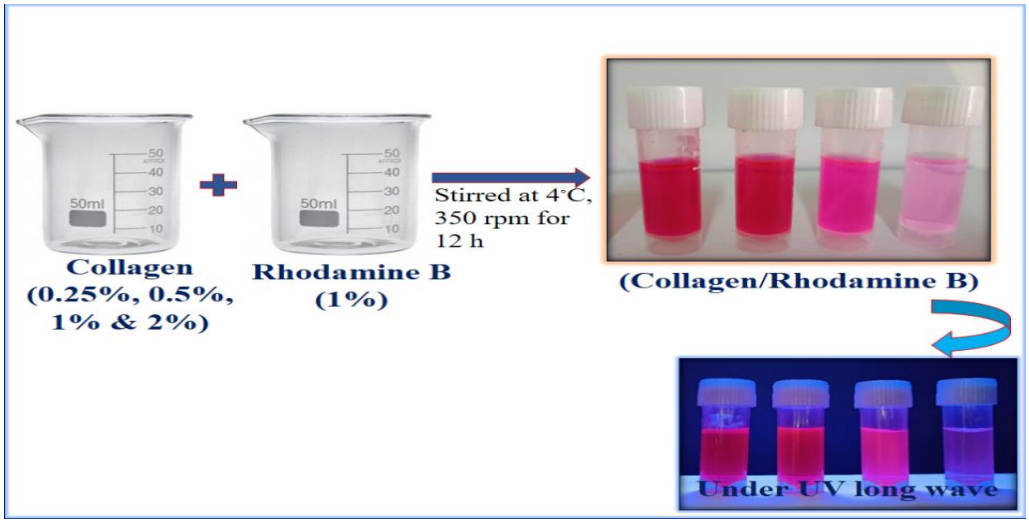

Fig. 6.3.1. The schematic representation for the preparation of different concentrations of collagen/Rhodamine B solution

6.3.2. RESULTS AND DISCUSSION

The present section deals with the preparation of solutions with different concentrations of collagen doped with Rhodamine B (1 %) which is a known fluorophore. The doped solution was analyzed by UV-Vis spectroscopy (UV-Vis) and photoluminescence (PL) for the determination of the quenching property of collagen. The intensity of the absorption of the dye decreased as the concentration of the collagen increased from 0.25 %-2 % **(Fig. 6.3.2.)** These results indicated the interaction of dye and collagen. In order to understand the actual interaction the emission spectra were recorded by photoluminescence (PL) method. The RB (1 %) showed emission spectra at 586 nm. When different concentrations of collagen were mixed with RB (1 %) the stokes shift observed in the emission maxima from 586 nm to 621 nm **(Fig. 6.3.3.)** could be due to the binding of RB to the hydrophobic pockets of collagen triple helix. The absolute quantum yield was decreased from 61.98 to 1.75 % as shown in **Fig. 6.3.4.** This sudden decrease in intensity was due to the quenching effect shown by collagen. This might be due to the intermolecular energy transfer between collagen with Rhodamine B molecule tends to decrease the intensity of fluorescent material. This opens up an additional application for Piscean collagen as a dye absorbent. This could be used as an absorbent for effluent cleaning purposes.

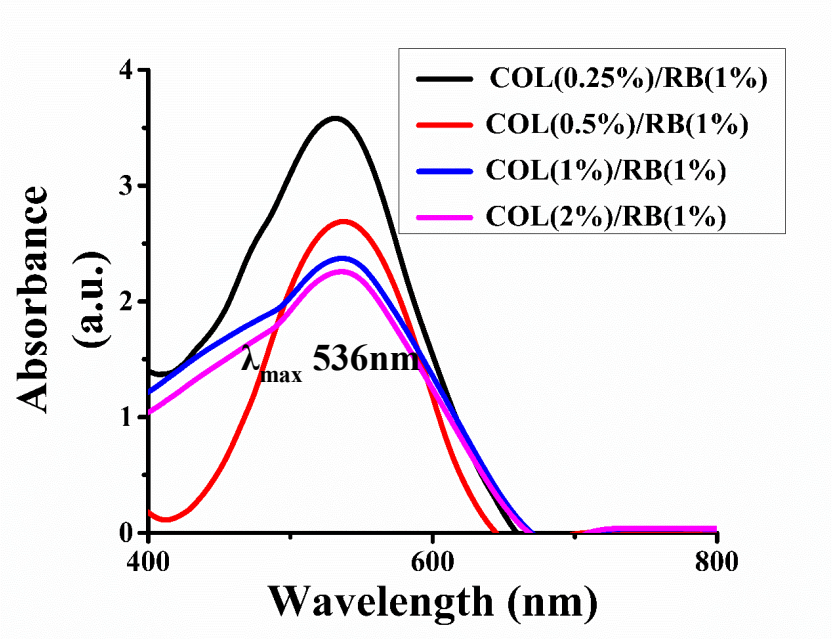

Fig.6.3.2. UV-Vis spectra of collagen/Rhodamine B solution

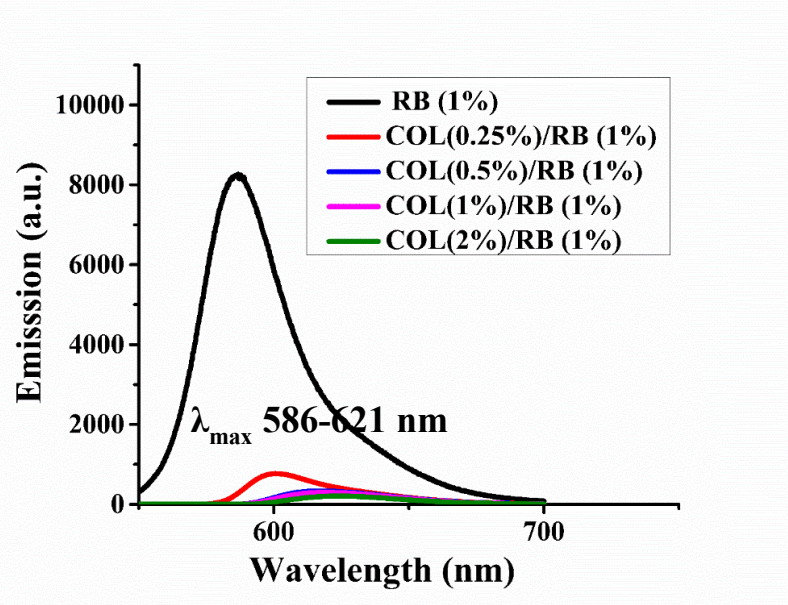

Fig. 6.3.3. Photoluminescence spectra of collagen/Rhodamine B solution

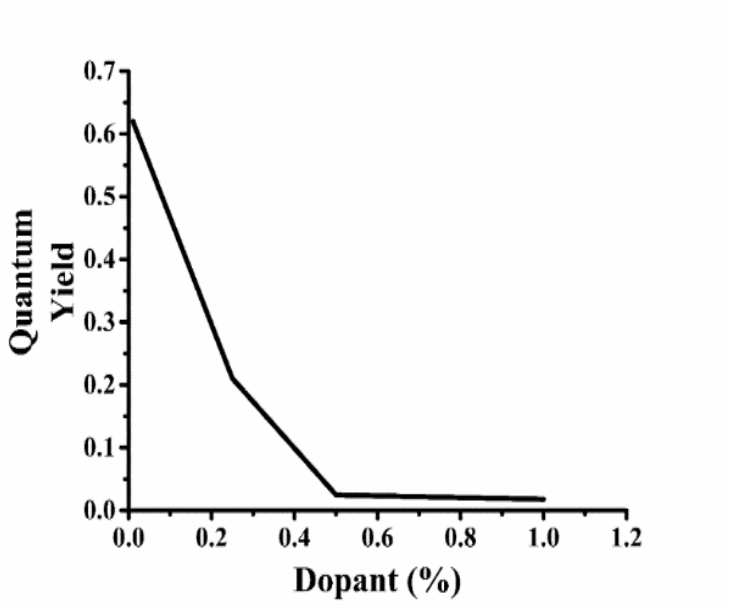

Fig. 6.3.4. Proportional decrease in the quantum yield of Collagen/ Rhodamine B solution with the dopant (%) increase

CONCLUSION

- Acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) from bone, skin, fins and head of Indian oil sardine and Indian mackerel were extracted by standard method and characterized
- The yield of ASC was higher than that of PSC. The yield of ASC from sardine skin (2.5 %) and mackerel skin (3.57 %) was higher compared to other parts of the fish
- The molecular weight of isolated collagen samples were ranging between 132 kDa-208 kDa was obtained from SDS-PAGE electrophoretic analysis
- The amino acid analysis exhibited the presence and composition of amino acids specific to type-I collagen. However the hydroxyproline content was seemed to be low which was compensated by higher amount of serine content, a characteristic of Piscean collagen
- The FESEM, UV and FTIR data confirmed the structure as type-I collagen for all isolated samples
- The ASC showed a maximum solubility at pH 4 and 4 % NaCl concentration due to the "salting in" effect
- The acid-soluble collagen isolated from skin of Indian mackerel was used for gel formulation with bioactive compound from papaya leaf extract. Results of *in vitro* cytotoxicity tests showed that prepared collagen gel did not induce a significant cytotoxic effect. The effective migration was observed in S4 (Collagen + glycerol + quercetin) with 25 μ g/ml concentration with 100 % migration of cells resulting in complete wound closure. As a result, collagen extracted from the skin of Indian mackerel could be used as an alternative biomaterial for wound dressing applications

- PVA/collagen nanofiber incorporated with TiO_2-NPs by electrospinning technique was developed for *in vitro* wound closure activity. Results of *in vitro* cytotoxicity tests on nanofibers revealed poor cytotoxic effect. The effective migration was observed in PVA/collagen/TiO_2-NPs (1 %) with 10 μ g/ml concentration with 65.45 % migration of cells resulting in wound closure. As a result, collagen extracted from the skin of Indian mackerel could be used as an alternative biomaterial for wound dressing applications

- Environmentally compatible biopolymer films of pullulan and collagen doped with different concentrations of ZnO-NPs were fabricated and could find use as edible packaging films. The nanocomposite films enhance antimicrobial activity, particularly it arrested the growth of fungus *A. niger.* Among tested films, the nanocomposite film with ZnO-NPs (0.5 %) dopant emerged as the best one. The penetration of nanoparticles into fungal cells greatly affected by nanoclusters formed in the polymer matrix. The nanocomposite film with ZnO-NPs (0.5 %) could be used as edible packaging material

- Studied on UVA/UVB filtering/protecting properties of PVA/collagen films doped with PBD and Bischalcone derivative (B1). The PVA/PBD/collagen (6 %) showed SPF value as 23.60 and UPF value as 52.38. The PVA/collagen/B1 (4 %) showed SPF value as 27.53 and UPF value as 58.23. A UPF of 50+ blocks out 98% of UV rays when worn. Hence in this study it was established that PVA/collagen/PBD and PVA/collagen/B1 composite films provide excellent alternative for UV-B and UV-A filtering materials
- The quenching property of isolated collagen was confirmed by using Rhodamine B dye. In the present study collagen decreased absolute quantum yield from 61.98 to 1.75 %. This sudden decrease in intensity was due to the quenching effect exhibited by collagen
- Hence, the isolation of versatile protein collagen from fish waste as well as its transformation into functional materials which are to be used in medicinal and materials fields has been embodied in the thesis

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List of publications

List of Research papers/Book chapter published in journals

1. **Niveditha, N.B.,** Sarojini, B.K. and Shrungeswara, A.H. (2020). A study on synergetic effect of papaya leaf active principles on wound healing capacity of collagen isolated from Indian mackerel. ***Journal of fisheries and environment.*** *44*(2): 24-36. ISSN: 2630-0702.
2. **Niveditha, N.B.,** Sarojini, B.K. and Suman Joshi D.S. Doddapaneni. (2020). Isolation and biochemical characterization of vital protein collagen: fish waste valorization of Arabian Sea fish *Sardinella longiceps* and its fluorescence quenching property. ***Biomedicine***. *40*(1): 36- 44. ISSN: 0970-2067.
3. **Niveditha, N.B.,** Sarojini, B.K., Suman Joshi D.S. Doddapaneni and Murari Mudiyaru Subrahmanya. (2021). Fabrication of environmentally compatible biopolymer films of pullulan/piscean collagen/ZnO nanocomposite and their antifungal activity. ***Journal of polymers and the environment.*** 29(4): 1192-1201. ISSN: 1566-2543. https://doi.org/10.1007/s10924-020-01953-y.
4. **Niveditha, N.B.,** Sarojini, B.K., Pushparekha and Kabiru. B. (2020). Customized eco-friendly UV-B filtering films from vital collagen isolated from Arabian Sea fish Indian oil sardine (*Sardinella longiceps*). ***In AIP Conference proceedings***. *2244(1):* 020003. https://doi.org/10.1063/5.0009427.
5. **Niveditha, N.B.,** Sarojini, B.K., and Pushparekha. (2021). Emerging trends in the synthesis, properties and applications of Nanogels derived from Pullulan, Collagen and Gelatin. In Nano Hydrogels 59-79. Springer, Singapore.

List of Research papers published in National/International conferences

1. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from Arabian Sea fish *Sardinella longiceps* in the National Conference on Natural bioactive compounds and potential effects on Health organized by Department of PG Studies and Research in Biochemistry, held on 27th January 2017. At St. Aloysius College, Mangalore.
2. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the fins of Arabian sea fish, *Sardinella longiceps*

presented at National Conference on Reaching and Unreached through science and technology: Recent advances in physical, chemical mathematical and biological sciences for energy, health and environment, Organized by Indian Science Congress Association at Mangalore University Mangalagangothri held from 8-9th September 2017.

3. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the skin of Arabian sea fish *Sardinella longiceps* presented at International Conference on emerging trends in chemical sciences (ICETCS) organized by Department Chemistry, Manipal Institute of Technology, Manipal, held from 14-16th September 2017.
4. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the head of Arabian Sea fish Indian mackerel (*Rastrelliger kanagurta*) presented at National Conference on Trends in bioactive natural products and health care Organized by Department of studies in Biochemistry at Chikkaluvara, Kodagu held from 6-7th October 2017.
5. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the bone of Arabian sea fish Indian mackerel (*Rastrelliger kanagurta)* at two days National conference on Advanced materials for health, energy and environment TEQIP-III organized by Department of Chemistry at Sri Jayachamarajendra College of Engineering, JSS Science and Technology University, Mysore, held from 23-24th March 2018 **(Best Poster Award- Second place).**
6. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the skin of Arabian Sea fish Indian mackerel (*Rastrelliger kanagurta*) presented at Indian council of chemists 37th Annual National conference organized by Department of Chemistry at National Institute of Technology Karnataka, Surathkal, Mangalore held from 12-14th December 2018.
7. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the wastes Arabian sea fish Indian oil sardine presented at the International Conference on advances in chemical and materials sciences organized by the Department of Chemistry, Mangalore University, Mangalagangothri, held from 17-18th October 2019.

8. **Niveditha, N.B.** and Sarojini, B.K. Studies on isolation and biochemical characterization of collagen from the total wastes Arabian sea fish Indian oil sardine presented at International conference on Advances materials and technology (ICMAT-20) organized by Department of Chemistry, at Sri Jayachamarajendra College of Engineering, Mysore, held from 16-18th January 2020.

National/ International Conference/Webinar attended

1. Attended two days workshop on "Research methodology for new research scholars" held during 4-5th March 2016 at Mangalore University, Mangalagangothri.
2. Attended National seminar and workshop on "Functional nanomaterials for energy, environment and health" held from 21-22nd March 2016 at Mangalore University, Mangalagangothri.
3. Participated in a one day National workshop on "Applied mass-spectrometry and recent advances in biomedical engineering" Organized by Biotek forum held on 19th November 2016, at P.A. College of Engineering, Konaje, Mangalore.
4. Attended National Workshop on "Emerging targets and biomarkers in cancer therapy" held during 16-18th May 2016 at Mangalore University, Mangalagangothri.
5. Attended Science Academies Lecture Workshop on "Recent trends in biological research: Health and disease perspective" held from 7-8th October 2016 at Mangalore University, Mangalagangothri.
6. Participated in a one day International Symposium on "Genomic instability and human diseases" Organized by Department of Applied Zoology held on 30th January 2017 at Mangalore University, Mangalagangothri.
7. Attended a week GIAN programme on "Nanofibers and their applications" Organized by Centre for Application of Radioisotopes and Radiation Technology, held from 27th February to 6th March 2017 at Mangalore University, Mangalagangothri.
8. Attended a week GIAN programme on "Lung inflammation and respiratory diseases" Organized by Department of studies in Biochemistry post-graduate center Jnana Kaveri, Chickkaluvara, held from 6-11th November 2017 at Mangalore University, Mangalagangothri.

9. Attended National Workshop on “Research ethics and intellectual property rights” Organized by Department of Industrial Chemistry and MSc. Course in Biochemistry, Mangalore University, Mangalagangothri, held on 1st March 2018.
10. Attended three days International Webinar Series on “Synthesis and characterization of nano materials and their novel applications” Organized by Department of physics, R.K. Valley, Rajiv Gandhi University of Knowledge Technologies, A.P. held from 17-19th August 2020.
11. Attended one day International Webinar on “Understanding tiny infectious agents-Viruses” Organized by Vijaya College, Mulki held on 28th August 2020.
12. Participated in a one day Webinar on “Ring size analysis of carbohydrate” Organized by Department of Chemistry and Biochemistry Sarada Vilas College K.M. Puram, Mysore held on 31st August 2020.
13. Attended one day Webinar on “Surface engineering of polymer membrane” Organized by Centre of Chemistry and Chemical Excellence held on 9th September 2020.
14. Attended one day National Webinar on “Computer aided drug discovery” Organized by Department of Chemistry, Manasagangotri, Mysore held on 14th September 2020.
15. Participated in a one day International Webinar on “Covid-19: Current status of vaccine development and Immunization” organized by Mangalore University and Microbiologists Society, India held on 30th September 2020.
16. Attended two day International Webinar on “Nanomaterials and flexible electronics” Organized by Mangalore University Incubation Centre held from 15th and 16th October 2020.
17. Participated in a Webinar on “Research options and opportunities in chemical sciences: Special emphasis on polymer science” Organized by Department of Chemistry, The National Institute of Engineering, Mysuru held on 19th October 2020.
18. Participated in a one day National Webinar on “Art of writing research paper” Organized by Government First Grade College and Centre for PG studies, Thenkanidiyur, Mangalore University Incubation Centre held on 20th October 2020.
19. Participated in a one day National Webinar on “Protection of innovations through patents and IPR” Organized by Karnataka State Akkamahadevi University, Vijayapura held on 28th October 2020.

20. Participated in a five day National e-Workshop on “Surface characterization: Tools and applications (SCTA 2020)” Organized by Department of Metallurgical and Materials Engineering, National Institute of Technology Karnataka, Surathkal held on 14th -18th December 2020.

Signature of the Research Guide Signature of the Research Scholar